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AU Horner, Anthony A.; Ronaghy, Arash; Cheng, Pei-Ming; Nguyen, Minh-Duc; Cho, Hearn J.; Broide, David; Raz, Eyal
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RAPID COMMUNICATION

Immunostimulatory DNA Is a Potent Mucosal Adjuvant[1]

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Most proteins delivered to mucosal surfaces fail to induce mucosal or systemic immune responses. We demonstrate that a single intranasal (i.n.) coadministration of a model antigen (β-galactosidase, β-gal) with immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) induces a mucosal IgA response equivalent to that induced by i.n. codelivery of β-gal with cholera toxin (CT). Furthermore, i.n. and intradermal (i.d.) deliv ry of the β-gal/ISS-ODN mix stimulates equivalent Th_1-biased systemic immune responses with high-lev l cytotoxic T lymphocyte (CTL) activity. In contrast, i.n. immunization with β-gal and CT results in a Th_2-biased systemic immune response with poor CTL activity. Our data show that i.n. delivery of ISS-ODN provides effective adjuvant activity for the induction of both mucosal and systemic Th_1-biased immune respons s. This immunization approach deserves consideration in the development of vaccines against muc sal pathogens. © 1998 Academic Press

Key Words: **immunostimulatory sequence DNA; muc sal adjuvant; IgA; vaccination.**

INTRODUCTION

The respiratory, gastrointestinal, vaginal, and rectal mucosa are sites where the majority of infectious agents are first encountered (1, 2). These surfaces are protected by secreted IgA (1–3). With intracellular pathogens, a CTL[3] response is important for elimination of the infectious agent (2, 4). Natural infection often induces these protective immune responses (1, 2). In contrast, delivery of monomeric protein antigens via mucosal routes generally does not stimulate any immune response, and delivery by systemic routes (i.e., i.d. and intramuscular, i.m.) leads to serum antibody production but mucosal IgA and CTL activity are not induced (1, 2). To produce a more comprehensive immune response to protein antigens, the use of adjuvants, including the mucosal adjuvant CT, has been explored.

Immunostimulatory sequence oligodeoxynucleotides (ISS-ODN) have previously been shown to provide effective adjuvant activity for the induction of systemic Th_1-biased immunity toward protein antigens coadministered via i.d. and i.m. routes (5–9). The immune response includes the induction of a Th_1 cytokine profile (IFN-γ but not IL-4), the production of high IgG2a and low IgG1 titers, and a CTL response (5–9). In this article we expand upon previous observations regarding the potent Th_1-biased adjuvant effect of ISS-ODN and demonstrate that, in addition, it is as good a mucosal adjuvant as CT. We show that i.n. administration of β-gal with either ISS-ODN or CT leads to equivalent mucosal IgA responses. In addition, i.n. and i.d. codelivery of β-gal with ISS-ODN induces equivalent Th_1-biased serum IgG subclass, splenic cytokine, and CTL responses, while i.n. β-gal/CT codelivery leads to a Th_2-biased systemic immune response. In considering the potential application of ISS-ODN as a vaccine adjuvant against mucosal pathogens, our data suggest that i.n. antigen/ISS-ODN delivery is superior to i.d. delivery for the induction of protective immunity.

MATERIALS AND METHODS

Immunization reagents. β-Gal and CT (Sigma, St. Louis, MO), ISS-ODN, and mutated phosphorothioate oligodeoxynucleotide (M-ODN) (Trilink Biotechnologies, San Diego, CA) were used to immunize mice. The ISS-ODN used in these studies has the following se-

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[3] Abbreviations used: i.n., intranasal; i.d., intradermal; i.g., intragastric; β-gal, β-galactosidase; ISS-ODN, immunostimulatory sequence oligodeoxynucleotide; M-ODN, mutated oligodeoxynucleotide; CT, cholera toxin; CTL, cytotoxic T lymphocyte; BALF, bronchoalveolar lavage fluid.

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quence 5′-TGACTGTGAACGTTCGAGATGA-3′. The M-ODN has the sequence 5′-TGACTGTGAACCTTAGAGATGA-3′.

Immunization protocols. Female BALB/c mice 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) and used in all experiments. Intranasal immunizations were performed with β-gal (50 μg) alone or mixed with 50 μg of ISS-ODN or M-ODN, or with CT (10 μg) in 30 μl of saline. Mice were anesthetized with Metofane (Mallinckrodt Veterinary Inc., Mundelein, IL) and 15 μl was delivered to each nare. Alternatively, mice received β-gal (50 μg) plus ISS-ODN (50 μg) in 50 μl of saline injected i.d. into the base of the tail, or β-gal (200 μg) plus ISS-ODN (50 μg) administered intragastrically (i.g.) by blunted needle in 400 μl of 0.2 M Na bicarbonate. Mice were fasted for 4 h before i.g. immunization.

Bronchoalveolar lavage and fecal IgA extraction. Bronchoalveolar lavage fluid (BALF) was obtained by cannulation of the trachea of sacrificed mice during week 7. The lungs were then flushed with 0.8 ml of PBS. The return was spun to remove cellular debris, and frozen at −70°C until IgA assay. Feces were collected at 2-week intervals and IgA was extracted according to a previously published protocol (10). Briefly, three to six pieces of freshly voided feces were collected and subsequently dried in a Speed Vac Concentrator. After feces were dried, net dry weights were recorded, and the material was resuspended in PBS with 5% nonfat dry milk and protease inhibitors at a ratio of 20 μl/mg of feces to standardize for variability in the amount of fecal material collected (10). The solid matter was resuspended by vortexing for 2 h followed by centrification at 16,000*g* for 10 min to separate residual solids from supernatant. Supernatants were then frozen at −70°C until IgA assay.

Immunologic assays. Serum, BALF, and fecal extraction fluid were used in ELISA assays for antigen-specific immunoglobulin as described previously (8, 9). Results are expressed in units/milliliter based on pooled high titer anti-β-gal standards obtained from mice receiving multiple immunizations. The undiluted fecal IgA and serum IgG standards were given arbitrary concentrations of 2000 and 400,000 U/ml respectively. Samples were compared to the standard curve on each plate using the DeltaSOFT II v. 3.66 program (Biometallics, Princeton, NJ). Mouse spleens were harvested at week 7 for CTL and cytokine assays. For CTL assays, 7×10^6 splenocytes from immunized mice were incubated with 6×10^6 mitomycin C-treated naive splenocytes in the presence of recombinant human IL-2 and class I $H2^d$-restricted β-gal nanopeptide (T-P-H-P-A-R-I-G-L) as previously described (9). After 5 days, restimulated cells were harvested and specific lysis of target cells measured (9). Splenocyte cytokine profiles were conducted by incubation of 5×10^5 splenocytes in 96-well plates in a final volume of 200 μl of supplemented RPMI 1640 with β-gal added at 10 μg/ml, at 37°C/5% CO_2 as previously described (8, 9). Culture supernatants were harvested at 72 h and analyzed by ELISA. A standard curve was generated using known amounts of recombinant IFN-γ (PharMingen, San Diego, CA) and IL-4 (Genzyme, Cambridge, MA). Each culture supernatant was compared to the standard curve on the plate using the DeltaSOFT II v. 3.66

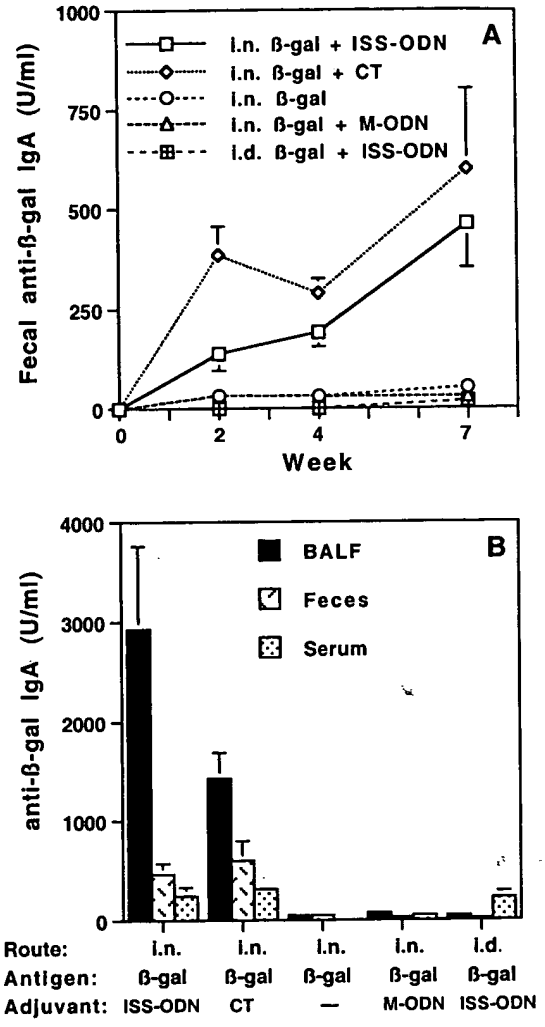

FIG. 1. IgA responses. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Results were obtained by ELISA and represent mean values for 4 mice per group. Error bars reflect the standard errors of the means. Results are representative of 3 similar and independent experiments. (A) Fecal IgA. Feces were collected at 2, 4, and 7 weeks and IgA extracted as described under Materials and Methods. There was no significant difference in anti-β-gal IgA levels between the i.n. β-gal/ISS-ODN and i.n. β-gal/CT vaccinated groups except at 2 weeks ($p = 0.03$). (B) BALF and serum IgA. BALF and serum were obtained at sacrifice during week 7 and compared to week 7 fecal IgA. There was no significant difference in the BALF anti-β-gal IgA levels between i.n. β-gal/ISS-ODN and i.n. β-gal/CT immunized groups.

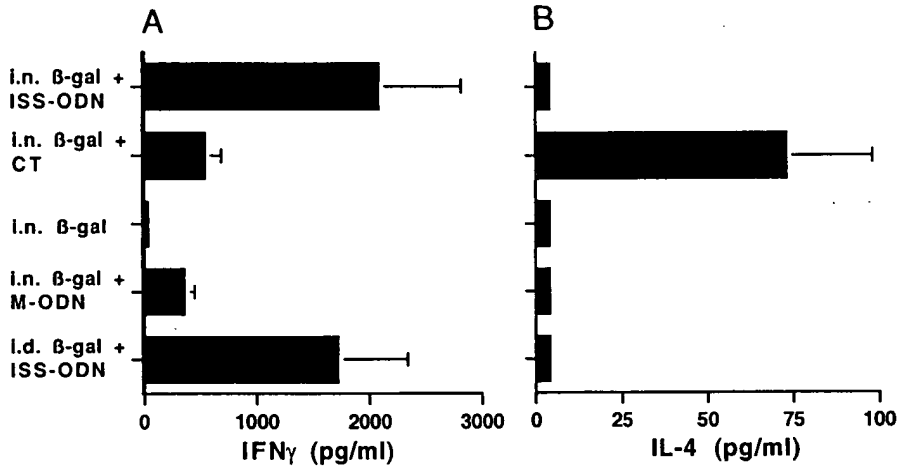

FIG. 2. Antigen-induced cytokine profiles. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Splenocytes were harvested from sacrificed mice during week 7 and cultured in media with or without β-gal (10 μg/ml), and 72-h supernatants were assayed by ELISA. Splenocytes cultured without β-gal produced no detectable IFN-γ or IL-4 (data not shown). Results represent the mean for 4 mice in each group and similar results were obtained in 2 other independent experiments. Error bars reflect standard errors of the means. (A) IFN-γ levels. IFN-γ levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically higher than in other immunization groups ($P = 0.05$ for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT vaccinated mice). (B) IL-4 levels. IL-4 levels above background were detected only in mice immunized with i.n. β-gal/CT ($P = 0.04$ verses background).

program. Statistical analysis of results was conducted using Statview computer software (Abacus Concepts, Grand Rapids, MI). A two-tailed Student t test was used to establish p values, and those ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

ISS-ODN is an effective mucosal adjuvant. Cholera toxin is the most potent known mucosal adjuvant (2). Therefore, the mucosal IgA response of mice immunized with i.n. β-gal/ISS-ODN and β-gal/CT were compared. As can be seen in Fig. 1, at 7 weeks post β-gal/ISS-ODN and β-gal/CT vaccination the mean anti-β-gal IgA levels were 462 and 599 U/ml in fecal material and 2935 and 1432 U/ml in BALF, respectively. Differences in mucosal IgA levels between i.n. β-gal/ISS-ODN-immunized and i.n. β-gal/CT-immunized mice were not statistically significant. To establish that a mucosal adjuvant was needed for the induction of mucosal IgA, we vaccinated mice i.n. with β-gal alone or with M-ODN. However, i.n. immunization without mucosal adjuvant resulted in no detectable IgA. We next evaluated whether contact with the respiratory mucosa was required for ISS-ODN to have mucosal adjuvant activity. Mice were therefore vaccinated with β-gal and ISS-ODN via i.d. and i.g. routes. These routes of immunization did not lead to measurable IgA in mucosal secretions (data for i.g. immunization not shown). To establish whether the IgA detected in fecal material and BALF of vaccinated mice was actively secreted by mucosal tissue or passively diffused from serum, anti-β-gal IgA levels in serum, fecal material, and BALF were compared. It should be noted that initial acquisition of BALF and fecal samples required an unmeasurable dilution of the IgA content of the material which does not occur when obtaining serum. Despite this fact, i.n. β-gal/ISS-ODN-immunized and i.n. β-gal/CT-immunized mice demonstrated higher levels of anti-β-gal IgA in feces and BALF then in serum, strongly suggesting that active secretion of anti-β-gal IgA from mucosal surfaces occurred in these mice (Fig. 1B).

These results demonstrate that ISS-ODN and CT have equivalent mucosal adjuvant activity with a test antigen that has no capacity to induce mucosal IgA production when delivered alone. In addition, we show that i.d. delivery of β-gal with ISS-ODN does not lead to a mucosal IgA response. Taken together these findings show that ISS-ODN is an excellent adjuvant for the induction of mucosal immunity when codelivered with antigen via the nose.

Immunization with β-gal and ISS-ODN by the i.n. route induces a vigorous Th_1-biased systemic immune response. We next evaluated the magnitude and phenotype of the systemic immune response induced by i.n. β-gal/ISS-ODN immunization. Splenocytes were harvested from mice 7 weeks after vaccination and incubated with β-gal. Culture supernatants were assayed for the production of IFN-γ and IL-4, cytokines classically associated with Th_1 and Th_2 immunity respectively (11, 12) (Fig. 2). Splenocytes from mice immunized with β-gal and ISS-ODN via the i.n. and i.d. routes produced a mean of 2084 and 1720 pg/ml of

IFN-γ, respectively (p value not significant), but no detectable IL-4. In contrast, i.n. vaccination with β-gal and CT led to splenocyte production of a mean of 542 pg/ml of IFN-γ and 73 pg/ml of IL-4 ($p = 0.05$ for both IFN-γ and IL-4 when compared to i.n. β-gal/ISS-ODN vaccination). Intranasal immunization with β-gal alone or with M-ODN led to much lower or undetectable cytokine production from splenocytes.

IFN-γ is a switch factor for IgG2a production, while IL-4 is a switch factor for IgG1 (11, 12). Given the splenic cytokine profiles, it would therefore be predicted that i.n. β-gal/ISS-ODN coadministration would lead to higher IgG2a and lower IgG1 levels then i.n. β-gal/CT codelivery. Indeed, we found that i.n. and id β-gal/ISS-ODN-immunized mice produced equivalent Th_1-biased serum antibody responses, whereas i.n. β-gal/CT vaccination led to a Th_2-biased IgG subclass profile.

At 7 weeks post-i.n. and i.d. β-gal/ISS-ODN immunization mean serum anti-β-gal IgG2a levels were 306,144 and 362,850 U/ml, and anti-β-gal IgG1 levels were 5971 and 3676 U/ml, respectively (Fig. 3). These differences were not statistically significant. In contrast, i.n. vaccination with β-gal and CT induced mean serum IgG2a and IgG1 levels of 94,518 and 36,471 U/ml ($p = 0.005$ for IgG2a and $P = 0.004$ for IgG1 compared to i.n. β-gal/ISS-ODN immunization). Again, i.n. immunization with β-gal alone or with M-ODN led to poor or undetectable IgG responses.

Cumulatively, these observations demonstrate that i.n. and i.d. delivery of antigen with ISS-ODN lead to equivalent Th_1-biased cytokine and antibody profiles, whereas i.n. β-gal/CT coadministration leads to a Th_2-biased systemic immune response. Considered in conjunction with the IgA data previously presented, we further demonstrate that production of mucosal IgA can occur in the context of both Th_1- and Th_2-biased systemic immune responses.

Codelivery of β-gal plus ISS-ODN by the i.n. route induces a strong splenic CTL response. Although development of antigen-specific CTL activity is associated with Th_1-biased immunity, not all Th_1-biased immune responses include the development of cytotoxic T cells (2, 4). Therefore, we next evaluated the ability of i.n. codelivery of β-gal and ISS-ODN to induce a CTL response. As demonstrated in Fig. 4, mice immunized with β-gal and ISS-ODN by either the i.n. or i.d. route displayed vigorous splenic CTL activity. At an E:T ratio of 5:1, i.n. and i.d. codelivery of β-gal/ISS-ODN led to 52 and 39% specific lysis of target cells, respectively. The difference was not statistically significant. However, i.n. β-gal/CT vaccination resulted in only 3% specific lysis at the same E:T ratio ($p = 0.005$ compared to vaccination with i.n. β-gal/ISS-ODN). Likewise, i.n. immunization with β-gal alone or with M-ODN led to poor or undetectable CTL responses. These results

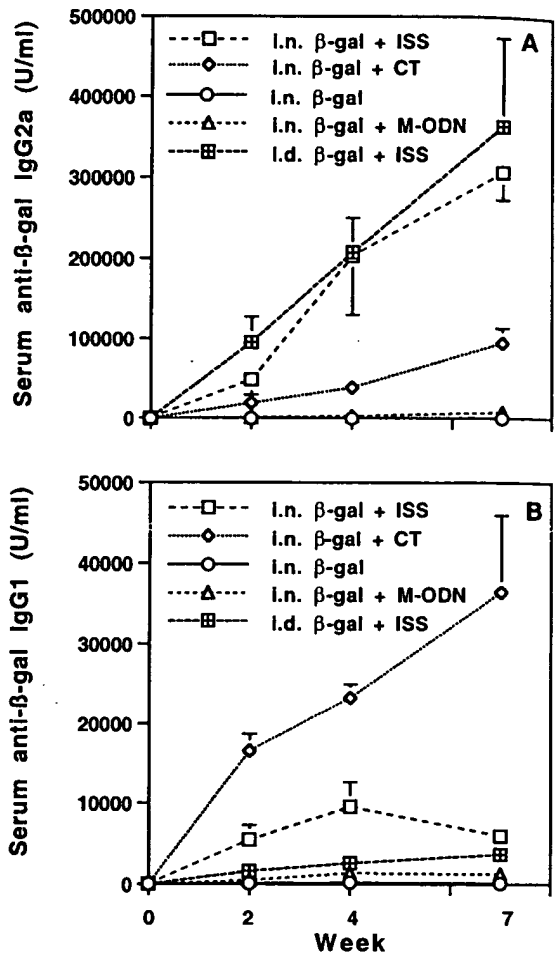

FIG. 3. IgG subclass profiles. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Serum was collected at 2, 4, and 7 weeks from immunized mice and assayed by ELISA. Results represent mean values for 4 mice per group, and error bars reflect standard errors of the means. Results are representative of 3 similar and independent experiments. (A) Serum IgG2a. Serum IgG2a levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically higher than in other immunization groups at 7 weeks ($p = 0.005$ for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT-vaccinated mice). (B) Serum IgG1. Serum IgG1 levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically lower than in i.n. β-gal/CT-immunized mice at all time points ($p = 0.003$, $p = 0.02$, and $p = 0.02$ for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT-vaccinated mice at 2, 4, and 7 weeks, respectively).

show that while i.n. and i.d. β-gal/ISS-ODN coimmunization leads to equivalent and robust CTL responses, i.n. β-gal/CT coadministration leads to a poor CTL response. In addition, the CTL assay results further demonstrate the dichotomy between the Th_1- and Th_2-biased systemic immune responses seen when β-gal is codelivered i.n. with ISS-ODN or with CT, respectively.

In summary, our findings demonstrate that i.n. delivery of antigen with either ISS-ODN or CT leads to an equivalent and vigorous mucosal IgA response, whereas i.d. codelivery of antigen with ISS-ODN does

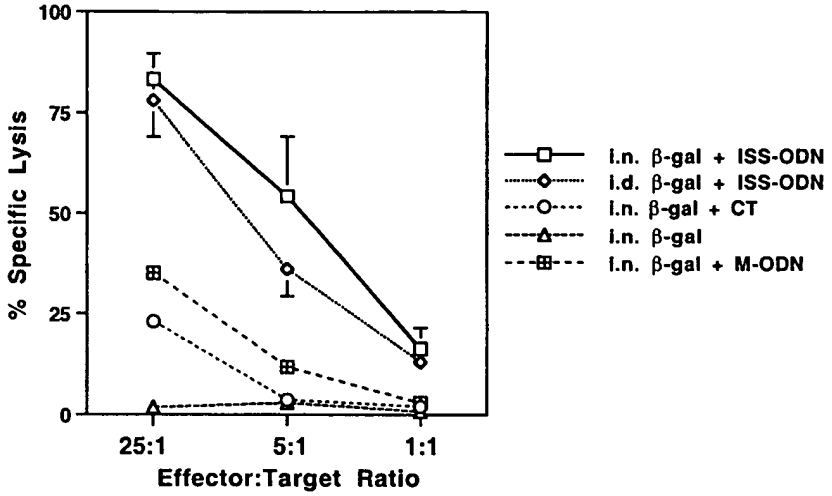

FIG. 4. CTL responses. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Splenocytes were harvested from mice at week 7 and CTL responses were determined as outlined under Materials and Methods. Results represent mean values for 4 mice per group, and error bars reflect standard errors of the means. Results are representative of 3 similar and independent experiments. CTL responses were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice at all E:T ratios, but statistically higher than in i.n. β-gal/CT-immunized mice at E:T ratios of 25:1 and 5:1 ($p = 0.005$ and $p = 0.05$ for i.n. β-gal/ISS-ODN versus in β-gal/CT-immunized mice at E:T ratios of 25:1 and 5:1, respectively).

not. However, i.d. and i.n. vaccination with β-gal and ISS-ODN induce equivalent systemic Th_1-biased immune responses characterized by high levels of antigen-specific IFN-γ but no IL-4 production from cultured splenocytes, high IgG2a and low IgG1 serum concentrations, and vigorous CTL responses. In contrast, i.n. codelivery of β-gal with CT leads to a Th_2-biased systemic immune response characterized by low IFN-γ but substantial IL-4 production from *in vitro* antigen-stimulated splenocytes, high IgG1 and low IgG2a serum concentrations, and a poor CTL response. The observation of equivalent mucosal IgA levels in the context of Th_1-biased and Th_2-biased systemic immune responses with i.n. β-gal/ISS-ODN and β-gal/CT immunizations respectively is consistent with other published results (18). Mariarosaria and colleagues recently demonstrated that oral delivery of tetanus toxoid with CT led to mucosal IgA production in conjunction with a Th_2 systemic immune profile and that coadministration with oral IL-12 skewed the systemic immune response toward a Th_1 phenotype, whereas mucosal IgA production was unaffected (18). Taken together, these findings document that synthesis of mucosal IgA can occur in the context of both Th_1- and Th_2-biased systemic immunity.

Mucosal IgA and CTL responses are known to provide protection against a number of infectious agents (1–3). HIV is but one example (4, 13, 14). There are a number of strategies available for the development of vaccines which induce these immune parameters. However, none at present appear globally applicable (15). Live attenuated vaccines produce robust immunity including mucosal IgA and CTL responses. Unfortunately, difficulty in attenuating many pathogens and the risk of iatrogenic disease limits the use and development live attenuated vaccines (1, 2, 13, 15). On the other hand, recombinant proteins from infectious agents are generally safe but induce relatively poor immune responses, and are not active when delivered to mucosal surfaces (1, 2). However, mucosal adjuvants can improve immune responses toward coadministered protein antigens substantially (1, 2, 15). Cholera toxin is an extremely potent mucosal adjuvant, but is inherently toxic and induces a Th_2-biased immune response that includes the development of IgE and allergic sensitization toward the target antigen (16, 17). At present, such toxicity and other technical problems have kept many adjuvants from becoming available for use in humans (15). Alum is essentially the only adjuvant in clinical use today. It is relatively weak, does not work with a number of antigens, does not induce CTL activity, and, because it must be delivered systemically, does not induce mucosal IgA (15). A safe and effective mucosal adjuvant would be of great value in the development of better vaccines. ISS-ODN is a potent adjuvant which works with a wide range of protein antigens, and generally induces a Th_1-biased immune response with CTL activity (5–9). In this report we have shown that both i.n. and i.d. administration of protein with ISS-ODN leads to vigorous Th_1-biased systemic immune responses, whereas only i.n. delivery induces a mucosal immune response. Therefore, i.n. delivery of relevant antigens with ISS-ODN may well prove superior to i.d. delivery for the induction of protective

immunity to mucosal pathogens. Our personal experience has been that ISS-ODN is easy to manufacture, stable, and without i.d.entified toxicity at immunogenic doses in mice and primates (unpublished observations). Additionally, use of antisense phosphorothioate oligodeoxynucleotides in monkeys and human clinical trials has demonstrated no significant toxicity with daily doses of up to fivefold more per kilogram than those used in the present study (19). Moreover, we and others have shown that human and mouse immunocytes display similar immunologic responses to ISS-ODN, suggesting that our present findings might also be applicable to humans (8, 20). The data presented represent a proof of principle which shows that in addition to its systemic adjuvant activity, ISS-ODN is an excellent mucosal adjuvant, and suggests a novel approach for the development of vaccines against infectious agents.

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